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PRINCIPAL INVESTIGATOR: Qiang Zhou, Ph.D.

CONTRACTING ORGANIZATION: Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

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Cambridge, Massachusetts 02139

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Control of transcriptional elongation has been recognized as an important step in gene regulation, but mechanisms regulating the efficiency of elongation by RNA polymerase II have not been extensively studied. HIV-1 Tat has been used as a model system to study this process. It has been suggested that polymerase elongation can be modulated by association with general transcription elongation factors such as Elongin and TFIIF. Elongin has been identified as a functional target of the von Hippel-Lindau tumor suppressor (VHL) protein. VHL binds tightly to Elongin and inhibits its activity. We have established a reconstituted transcription reaction that supports a Tat-specific and TAR-dependent activation of HIV-1 transcription. Using this system we have previously identified and purified a Tat-specific cofactor Tat-SF1. The reconstituted reaction also contains general elongation factors such as TFIIF and Elongin. We found that increasing the concentration of recombinant Elongin, but not TFIIF, in transcription reactions suppressed the degree of Tat activation by increasing the efficiency of elongation independent of Tat. Our data are consistent with a model that polymerase elongation in vitro may be limited by an Elongin-mediated process and Tat stimulates elongation by facilitating the interaction of Elongin with elongating polymerase complexes.

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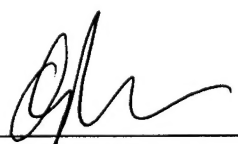

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Introduction

Control of transcriptional elongation has been recognized as an important step in gene regulation, but mechanisms regulating the efficiency of elongation by RNA polymerase II have not been extensively studied. For many eukaryotic genes (e.g. proto-oncogenes c-myc and c-fos), regulation of the processiveness of elongation is a major control step in gene expression (Bentley, 1995; Greenblatt et al., 1993; Krumm et al., 1993; Lis and Wu, 1993). The necessity for strict control of elongation is further highlighted by the finding that an elongation factor, Elongin, is probably the functional target of the von Hippel-Lindau tumor suppressor protein (Aso et al., 1995; Duan et al., 1995; Kibel et al., 1995). For the human immunodeficiency virus (HIV), regulation of polymerase elongation on HIV proviral DNA by Tat is absolutely essential for viral replication. Because the proteins that regulate elongation in many cellular and viral genes have not been identified, HIV-1 Tat has been used as a model system to study this process.

Tat, one of the regulatory proteins encoded by the HIV-1 genome, is essential for HIV gene expression and productive viral infection. In the absence of Tat, the HIV-1 LTR generates short transcripts. The presence of Tat, however, results in a large increase in the level of transcripts that extend through the 9.0-kb HIV genome. Tat stimulation of the efficiency of transcriptional elongation is primarily responsible for this dramatic increase in the level of full-length HIV transcripts (Cullen, 1990; Frankel, 1992; Gaynor, 1995; Jones and Peterlin, 1994; Karn and Graeble, 1992). Tat stimulates elongation by recognizing the trans-acting-response (TAR) RNA element. Located at the 5' end of the nascent viral transcript, TAR forms a stem-loop structure. The specific binding of Tat to TAR is primarily dependent on the 3-nt bulge and immediately flanking sequences in the double-stranded RNA.

Tat activation of HIV transcription is believed to be modulated by cellular co-factors that specifically recognize TAR or Tat, or both (Gaynor, 1995; Jones and Peterlin, 1994). It is known that sequences in the apical loop of TAR are important for Tat activation in vivo, because mutations in the loop completely blocked Tat trans-activation (Feng and Holland, 1988; Garcia et al., 1989; Selby et al., 1989). Furthermore, introduction of wild-type TAR RNA, but not TAR loop mutants, into a reconstituted transcription reaction containing excess Tat competes for a limiting component necessary for Tat activation (Zhou and Sharp, unpublished). These results suggest the existence of a limiting factor that may modulate Tat activation by recognizing TAR dependent on the wild-type loop sequence. We have observed that mutations in the apical loop altered the conformation of TAR around the bulge and the double-stranded RNA stem (Zhou and Sharp, unpublished). These changes may result in a decreased binding of the limiting factor to TAR.

Biochemical and genetic analyses of Tat activation of HIV transcriptional elongation have indicated the existence of cellular factors that affect Tat activity by directly contacting the Tat polypeptide (Jones and Peterlin, 1994). Tat-binding proteins have been isolated either by affinity chromatography on columns containing immobilized Tat protein, by probing a human expression library with purified Tat protein, or by the yeast two hybrid system (reviewed in Greenblatt et al., 1993; Jones and Peterlin, 1994; Gaynor, 1995). As yet, none of these proteins have been shown to be essential for Tat trans-activation.

Tat stimulation of HIV transcription has been shown to be cell type- and species-specific. Tat trans-activates well in many primate cell types but functions poorly in Drosophila, yeast, rodent and Chinese hamster ovary (CHO) cells (Hart et al., 1993; Jones and Peterlin, 1994; Newstein et al., 1990). It is postulated that certain cellular factors essential for Tat function are present in many primate cells, which are permissive to Tat function, but not in some non-permissive human cell types or cells of other species. The cellular factors responsible for the cell- and species-specific function of Tat could well be the factors that interact with TAR or Tat (Alonso et al., 1992; Hart et al., 1993). Consequently, the identification and characterization of these Tat cofactors will facilitate our understanding of the specificity of Tat-TAR interaction, the cell type and species specificity of Tat activation, and ultimately the mechanism of Tat stimulation of HIV transcriptional elongation.

A predominant form of Tat in HIV-1 infected cells is an 86-amino acid protein derived from two-exon mRNAs. Four important regions have been identified in Tat (Jones and Peterlin, 1994). (1) The amino-terminal region has been proposed to fold into an amphapathic α-helix frequently found in the activation domains of many transcription factors. (2) Sequences between amino acids 22-37 consist of a cysteine-rich region with seven highly conserved cysteine residues. (3) The hydrophobic core region between amino acids 38-47 is highly conserved among related immunodeficiency viruses, and the amino acid residues lysine41 and leucine43 are absolutely required for activity (Rice and Carlotti, 1990a). (4) A stretch of nine basic amino acids from 49 to 57 constitutes the basic domain, which mediates the binding of Tat to TAR RNA and is also required for nuclear localization. Tat binds TAR primarily through electrostatic interactions between the basic domain and the phosphate groups surrounding the TAR bulge. The hypothetical cellular TAR-binding factor may function by enhancing the specificity of this interaction. Mutagenesis and domain-swapping experiments indicate that the Cys-rich, the core, and the amino-terminal regions of Tat form an independent trans-activation domain (Rice and Carlotti, 1990a; Rice and Carlotti, 1990b). The recent NMR structure of Tat indicates that the Cys-rich region

and the basic domain are organized around the hydrophobic core and are exposed for interactions with other proteins and TAR RNA (Bayer et al., 1995).

Experimental methods, results and discussion.

This report covers the period from Aug. 1, 1996 to Jan. 31, 1997. Due to my transfer from MIT to the University of California, Berkeley at the end of Jan. 1997, the research project funded by this subject grant was suspended. I requested permission from the Army to transfer the balance of the grant to UC Berkeley, and this request was approved in the Spring of 1998. The new project period began on June 1, 1998 and will end on May 31, 2000.

In 1996 and 1997, a prevalent model in the Tat field proposed that polymerase elongation can be modulated by phosphorylation of the heptapeptide repeats within the COOH-terminal domain (CTD) of RNA polymerase II, or by association with a transcription elongation factor such as Elongin (SIII). CTD phosphorylation coincides with the passage of the paused RNA polymerase into a state competent for elongation (Dahmus, 1995; O'Brien et al., 1994). CTD has been shown to be phosphorylated by the basal transcription factor TFIIH, which is tightly associated with the Cdk-activating kinase CAK (Serizawa et al., 1995). Recently, it has been reported that a kinase-cyclin pair SRB10/11 associated with a unique subfraction of RNA polymerase II (holoenzyme) is also involved in CTD phosphorylation (Liao et al., 1995).

The recently discovered elongation factor, Elongin (SIII), is a complex consisting of a catalytic subunit A and two regulatory subunits B and C (Aso et al., 1995). It activates elongation by contacting polymerases and/or nascent RNA and suppressing polymerase pausing. Elongin was recently identified as a functional target of the von Hippel-Lindau tumor suppressor (VHL) protein (Duan et al., 1995; Kibel et al., 1995). VHL binds tightly to Elongin subunits B and C and inhibits its activity. In von Hippel-Lindau disease, mutations in VHL disrupt its interaction with Elongin subunits, resulting in excess Elongin and probably uncontrolled expression of certain oncogenic genes that are responsible for a variety of tumors. This suggests that the interference of VHL with Elongin's transcriptional elongation activity is perhaps the basis of its tumor-suppressor function (Krumm and Groudine, 1995).

I have previously developed a partially reconstituted in vitro transcription reaction that is Tat-specific and TAR-dependent for activation of HIV transcriptional elongation (Zhou and Sharp, 1995). Using this as a functional assay, I have previously identified and partially purified a HeLa nuclear activity, Tat-SF1 (for Tat stimulatory factor 1), that is essential for Tat activation of HIV transcription (Zhou and Sharp, 1995). This is the first identification of a distinct cellular activity that is essential for Tat

activation of HIV transcription but dispensable for the function of other activators. In the presence of Tat-SF1, Tat stimulates the efficiency of elongation by RNA polymerase II, whereas Sp1 and other DNA sequence-specific transcription factors activate the rate of initiation, independent of Tat-SF1.

In addition to Tat-SF1 activity, Tat stimulation of HIV transcription in the reconstituted reaction also requires a phosphocellulose 0.5-1.0 M KOAc fraction of HeLa nuclear extract (the pc-D fraction), and the purified basal factors TFIID, TFIIA, and transcription factor Sp1. The pc-D fraction contains several basal transcription factors (TFIIB, IIE, IIF, and IIH) and RNA polymerase II. Because pc-D can not be substituted with highly purified basal transcription factors, it probably contains other activities necessary for Tat function. Immunoblotting analysis indicated that pc-D fraction also contains elongation factor Elongin.

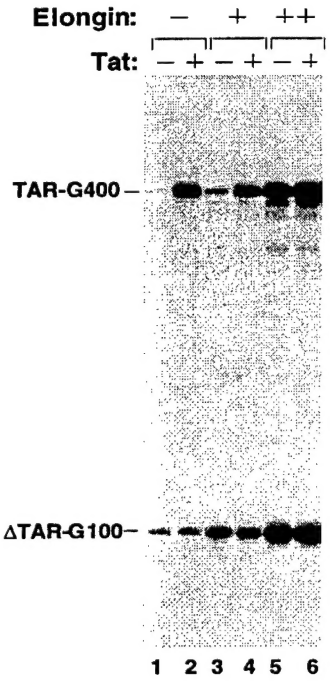

Fig. 4. High levels of recombinant Elongin suppress Tat trans-activation in vitro.

Reconstituted in vitro transcription reactions were carried out in the presence (+) or absence (-) of Tat as described (Zhou and Sharp, 1995). An HIV template with wild-type TAR (TAR-G400) and an internal control template (ΔTAR-G100) with a mutant TAR were included in a single reaction. No recombinant Elongin was added to the first two reactions. High levels of recombinant Elongin increased transcription from both TAR(+) and TAR(-) templates in the absence of Tat (compare lane 1 with lanes 3 and 5) and suppressed Tat activation (compare lanes 1 & 2 with lanes 3-6)

If Tat stimulated elongation by enhancing the effect of one of the known transcription elongation factors, then increasing the concentration of this factor could suppress the degree of Tat activation by increasing the efficiency of elongation independent of Tat. In agreement with this hypothesis, increasing the concentration of recombinant Elongin (kindly provided by Conaway's laboratory) in transcription reactions was found to suppress the degree of Tat activation by increasing the efficiency of elongation independent of Tat (Figure 1). It is likely that high concentrations of Elongin facilitated the association of this elongation factor with polymerase elongation complexes and thereby bypassed the requirement of Tat for efficient elongation *in vitro*.

On the other hand, polymerase elongation in vitro is probably limited by an Elongin-mediated process and Tat stimulates elongation perhaps by facilitating the interaction of Elongin with elongating polymerase complexes.

Previously, it has been reported that increased levels of a partially purified TFIIF fraction suppressed Tat activation by increasing elongation under Tat(-) conditions (Kato et al., 1992). We could not confirm this observation with highly purified or recombinant TFIIF. Furthermore, our data indicate that unlike Elongin, none of the transcription factors TFIIA, B, E, F, H nor RNA polymerase II can suppress the degree of Tat activation when present at high concentrations (Zhou and Sharp, 1995).

Conclusions

The results described above and in our previous paper (Zhou and Sharp, 1996) are consistent with the model that Tat stimulates the efficiency of HIV transcriptional elongation by recruitment of the Tat-SF1-kinase complex to the HIV promoter through a Tat-TAR interaction. Once recruited, Tat-SF1 or its associated kinase could modulate the conformation or composition of the elongating polymerase complex, facilitate the interaction of Elongin with the polymerase, or directly affect the phosphorylation of the components within the complex. Although Elongin was identified as a general elongation factor, polymerase elongation in vitro is probably limited by an Elongin-mediated process and Tat stimulates elongation perhaps by facilitating the interaction of Elongin with elongating polymerase complexes. Future studies will be directed at examining possible interactions between Tat and Elongin and between Elongin and RNA polymerase II.

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